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Epigenetic Down-Regulation of ARF Expression Is a Selection Step in Immortalization of Human Fibroblasts by c-Myc

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Abstract

The transcription factor c-Myc is implicated in the pathogenesis of many cancers. Among the multiple functions of c-Myc, activation of *hTert* and other genes involved in cellular life span contributes to its role as an oncogene. However, the ability of c-Myc to directly immortalize human cells remains controversial. We show here that overexpression of c-Myc reproducibly immortalizes freshly isolated human foreskin fibroblasts. c-Myc–immortalized cells displayed no gross karyotypic abnormalities but consisted of an oligoclonal population, suggesting that additional events cooperated to achieve immortalization. Levels of p53 and p16 were increased, but both p53-dependent DNA damage response and growth arrest in response to p16 overexpression remained intact. A marked decrease in expression of the tumor suppressor ARF occurred in several independently established c-Myc–immortalized cell lines. Methylation-specific PCR showed that the *ARF* gene was methylated in immortalized but not early-passage c-Myc cells, whereas *p16* was unmethylated in both cell populations. Restoration of *ARF* expression by treatment with a demethylating agent or overexpression by a retroviral vector coincided with inhibition of proliferation and senescence of c-Myc–immortalized cells. Our findings predict that epigenetic events play a significant role in human tumors that express high levels of c-Myc. (Mol Cancer Res 2007;5(11):1181–9)

Introduction

The *c-Myc* oncogene has been implicated in the pathogenesis of many human tumors (1). The c-Myc protein is a basic helix-loop-helix leucine zipper transcription factor that modulates expression of a cohort of genes that function to promote cell growth and cell cycle entry, sensitize cells to apoptosis, and interfere with differentiation (2-4). c-Myc has the potential to influence polymerases I and III genes in addition to polymerase II, a property that explains the dramatic stimulation of cell growth by c-Myc (5). More recently, a global regulation of chromatin structure by c-Myc through spreading of acetylation at sites distant from c-Myc–binding sites has been shown (6), indicating that c-Myc overexpression might have profound and generalized effects on cell behavior through epigenetic modification.

Here, we chose to address the controversial role of c-Myc as an immortalizing gene, a function that may be in part explained by its ability to regulate genes influencing the life span of human cells, including *WRN* (7) and *hTert*, the catalytic subunit of telomerase (8-11). The historical definition of c-Myc as an immortalizing gene was originally derived from experiments indicating the cooperation between c-Myc and Ras in transformation of rodent cells (12), where c-Myc seemed essential for the immortalization but not for anchorage-independent properties of the transformed cells. Rodent cells immortalized by c-Myc characteristically inactivate the ARF/p53 pathway by loss of either functional p53 or ARF (13). The *INK4a/ARF* locus encodes two tumor suppressors, *p16* and *ARF*, and undergoes frequent disruption in human cancers (14, 15). ARF stabilizes p53 in a regulatory feedback loop (16-19) and is up-regulated in response to aberrant expression of several oncogenes, including c-Myc (13, 20).

In contrast, data on the ability of c-Myc to immortalize human cells are scarce as reports have described the isolation of rare immortal clones from human fibroblast cultures transfected with either c-Myc or v-Myc (21, 22). The rarity of immortal clones could have been due to poor transfection efficiency of the human fibroblasts as well as the fact that human fibroblasts, in contrast to murine fibroblasts, are notoriously resistant to immortalization (23). More recently, immortalization of human

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prostate epithelial cells with c-Myc has also been reported (24). Although these early results suggested c-Myc could immortalize human cells, more recent studies showed acute activation of c-Myc using an estrogen-inducible form of the protein (MycER) could induce a cell cycle arrest in human fibroblasts (25), and constitutive c-Myc induced a p16-dependent arrest, which was abolished in a p16-deficient cell line (26). This latter study indicates that the basal cellular level of p16 is important and that culture-imposed stress, including increased p16 over time in culture, may play a factor in determining cellular response to oncogene expression (27).

To reexamine the immortalization potential of c-Myc in a more physiologic setting, we used freshly isolated human foreskin fibroblasts (HFF). HFFs express lower levels of p16 than other strains of human fibroblasts as they have been exposed to less culture-imposed stress and are resistant to senescence induced by oncogenic H-Ras (28). Thus, HFFs may be more representative of cells *in vivo* relative to other cultured cell lines. In addition, telomere length is the only factor that limits the life span of HFFs and they can be immortalized directly by expression of exogenous hTert (29). We also used a retroviral vector that overexpressed c-Myc constitutively and at similar levels to human tumors where c-Myc has been implicated, thus avoiding possible effects of acute Myc activation via the MycER chimera.

Our study shows that constitutive c-Myc overexpression reproducibly immortalizes HFFs. Interestingly, although c-Myc–immortalized HFFs accumulated increased levels of the tumor suppressors p53 and p16, they had markedly reduced to undetectable levels of ARF. Bisulfite modification confirmed epigenetic modification of the *ARF* gene by DNA methylation in immortalized but not early-passage c-Myc cells. In addition, restoration of *ARF* expression by treatment with the demethylating agent 5-aza-2′-deoxycytidine (5azaCdR) or overexpression of ARF following retroviral transduction coincided with impaired proliferation and an increase in senescence markers in c-Myc–immortalized cells. These observations suggest that loss of ARF, but not p53 or p16, is an important determinant of the immortalization of human fibroblasts by c-Myc.

Results

Immortalization of Human Fibroblasts by c-Myc

Early-passage HFFs were transduced with retroviruses expressing c-Myc or an empty vector control (pB). Following selection, pB-expressing control cells underwent ~80 population doublings before reaching senescence, whereas c-Myc–expressing cells bypassed senescence and continued to divide (Fig. 1A). Interestingly, proliferation of c-Myc–expressing cells slowed at the same time point that control cells approached senescence. At this inflection point, the c-Myc HFF population seemed heterogeneous, but after ~3 weeks, rapidly dividing cells took over the culture. c-Myc–expressing cells have currently undergone >200 population doublings. This ability of c-Myc to immortalize HFFs is reproducible, as we have been consistently able to establish immortalized cell lines with HFF isolates derived from four independent donors (Supplementary Fig. S1; data not shown).

We next examined whether c-Myc activated expression of telomerase in HFFs. Telomerase activity was detected in early-passage c-Myc–expressing cells (time point 1), decreased during middle passages (time points 2 and 3), and increased again in cells that emerged after the inflection point (time point 5; Fig. 1B). These variations in telomerase activity correlated in part with changes in c-Myc expression levels, which decreased as cells were passaged but were elevated again in the immortalized population (Fig. 1C).

In general, the levels of p53 and p16 increased in both control and c-Myc–expressing cells as they aged in culture (Fig. 1C). Interestingly, the levels of p53 and p16 were markedly higher in the late-passage c-Myc–expressing cells compared with control cells and early-passage c-Myc–expressing cells. However, the increased levels of p53 and p16 proteins were not sufficient to cause arrest because c-Myc–immortalized HFFs continued to divide at a rate similar to early-passage controls (Fig. 1A). Moreover, whereas control cells adopted a senescent morphology and stained positive for senescence-associated β-galactosidase (SA β-gal) activity by 161 days following selection, c-Myc–expressing cells did not seem senescent, were actively dividing, and remained primarily negative for SA β-gal activity (Fig. 1D).

We knocked down c-Myc by small interfering RNA in c-Myc–immortalized HFFs to confirm that overexpression of c-Myc directly facilitates immortalization (Fig. 2A). When c-Myc expression was suppressed, cells arrested, adopted a senescent morphology, and stained positive for SA β-gal activity (Fig. 2B). These observations indicate that c-Myc is necessary for continued proliferation and survival of our immortalized cells.

Selection of an Oligoclonal Population

The slow growth phase in the growth curve of c-Myc–immortalized HFFs indicated the possible clonality of c-Myc–immortalized HFFs. Therefore, we did Southern blot analysis with the retroviral vector (Fig. 3A) used for the transduction as probe. In early-passage cells, the digestion pattern obtained with either single cutter or no-cutter enzymes of the predicted integrated provirus showed an expected "smear" indicative of a polyclonal population where proviral DNA integrated at different sites in each cell. In contrast, c-Myc–immortalized cells exhibited a few individual bands, indicating the selection of an oligoclonal population during the immortalization step (Fig. 3B). However, this selection did not coincide with the acquisition of a stable karyotypic aberration because no gross chromosomal changes were detected in c-Myc–immortalized cells (Fig. 3C).

c-Myc–Immortalized HFFs Have an Intact p53-Dependent DNA Damage Response

Because the ARF/p53 pathway is commonly inactivated in c-Myc–immortalized rodent cells (13), we tested to see if the increased p53 protein in c-Myc–expressing cells (Fig. 1C) was functional by assessing the response to DNA damage. Following Adriamycin treatment to induce DNA damage, all cell populations arrested in G_1 and G_2 phases of the cell cycle (Fig. 4A) and levels of p53 and its downstream target p21 were induced (Fig. 4B). Furthermore, Rb protein was hypophosphorylated and levels decreased, consistent with cell cycle arrest. Similar results were obtained with a different, independently

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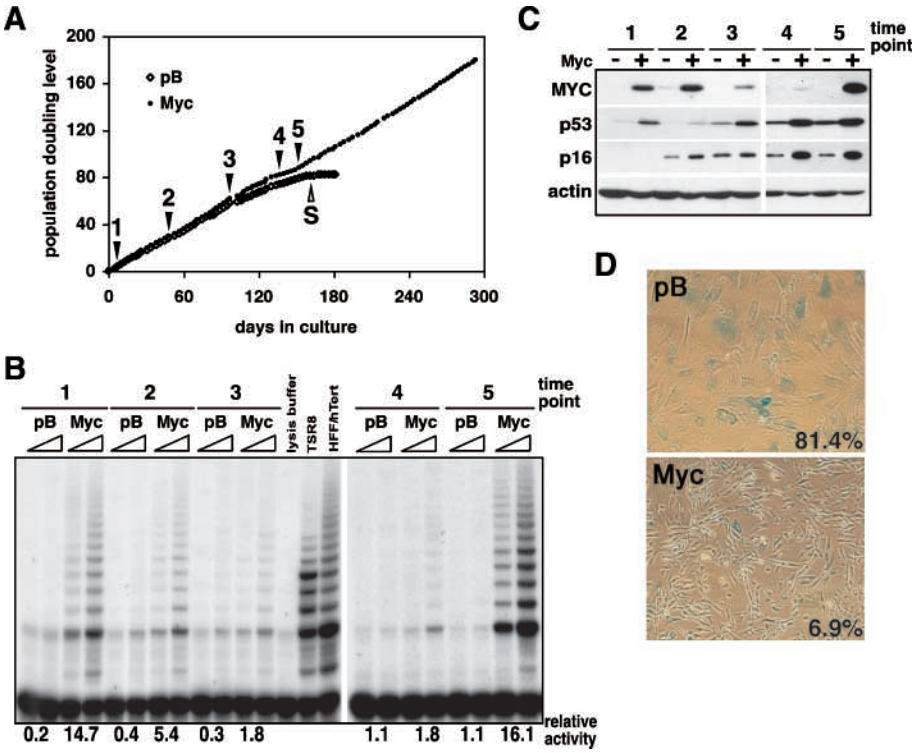

FIGURE 1. Immortalization of HFFs by c-Myc. **A.** Growth curve of pB-expressing (◇) and c-Myc–expressing (●) HFFs showing population doubling levels over time. Numbers (*1-5*) indicate time points when cells were analyzed for telomerase activity and protein levels. Cells were fixed and stained for SA β-gal activity on day 161 (*S*). **B.** Telomerase activity in pB-expressing and c-Myc–expressing HFFs. TRAP assay using 0.2 and 2 μg of cell extract from pB and c-Myc cells at the time points indicated. Lysis buffer represents a negative control and lysate from hTert-transduced HFFs and TSR8 are positive controls. Activity was normalized to the TSR8 control. **C.** Western blot analysis of protein lysates from pB (–) and c-Myc (+) cells collected at the indicated time points. Levels of c-Myc, p53, p16, and actin. **D.** SA β-gal staining of cells on day 161. pB cells were at population doubling level 82 and c-Myc cells at population doubling level 94. Percentages of cells staining positive.

established c-Myc–immortalized cell line (data not shown). These data indicate that all of the cell populations tested, including the c-Myc–immortalized cells, retain functional p53 as shown by intact p53-dependent response to DNA damage.

Analysis of INK4 Proteins p16 and p15 in c-Myc–Immortalized HFFs

Prostate epithelial cells immortalized with c-Myc have been shown to inactivate the p16 pathway (24), and we evaluated whether this occurred in c-Myc–immortalized HFFs. Transduction of all cell populations with p16-expressing retroviruses (Fig. 5A) caused arrest of cell proliferation and the appearance of cells that stained positive for SA β-gal activity (Fig. 5B), indicating that in c-Myc–immortalized cells p16 is still capable of mediating growth arrest.

Because Myc has been shown to suppress expression of p15INK4B (30), we analyzed p15 expression in c-Myc–immortalized HFFs. Comparing c-Myc–overexpressing HFFs with vector control HFFs, no detectable difference in the levels of p15 were observed (Fig. 5C). This indicates that down-regulation of p15 is not a necessary event in immortalization of human fibroblasts by c-Myc.

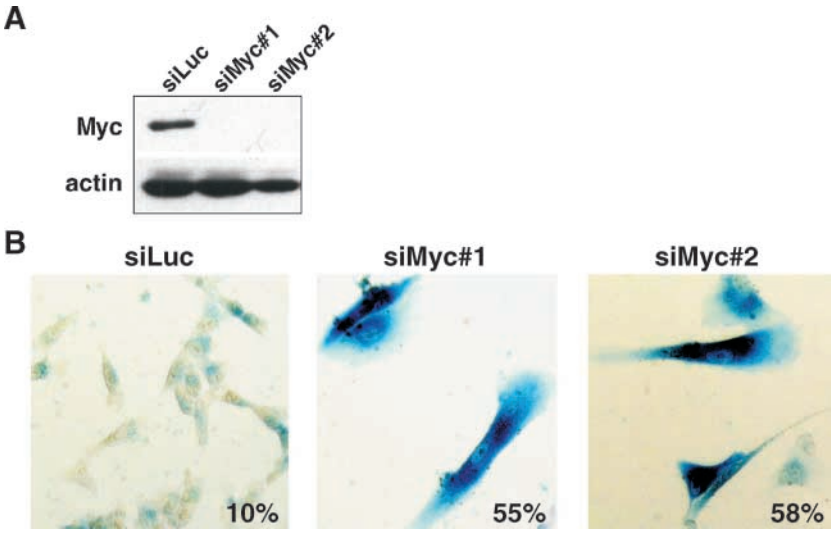

FIGURE 2. Knockdown of c-Myc induces senescence. **A.** Western blot analysis of protein lysates from c-Myc–immortalized cells transfected with small interfering RNA against luciferase as control (*siLuc*) or against c-Myc (*siMyc#1* and *siMyc#2*). Levels of c-Myc and actin. **B.** SA β-gal staining of cells described in **A**. Percentages of cells staining positive.

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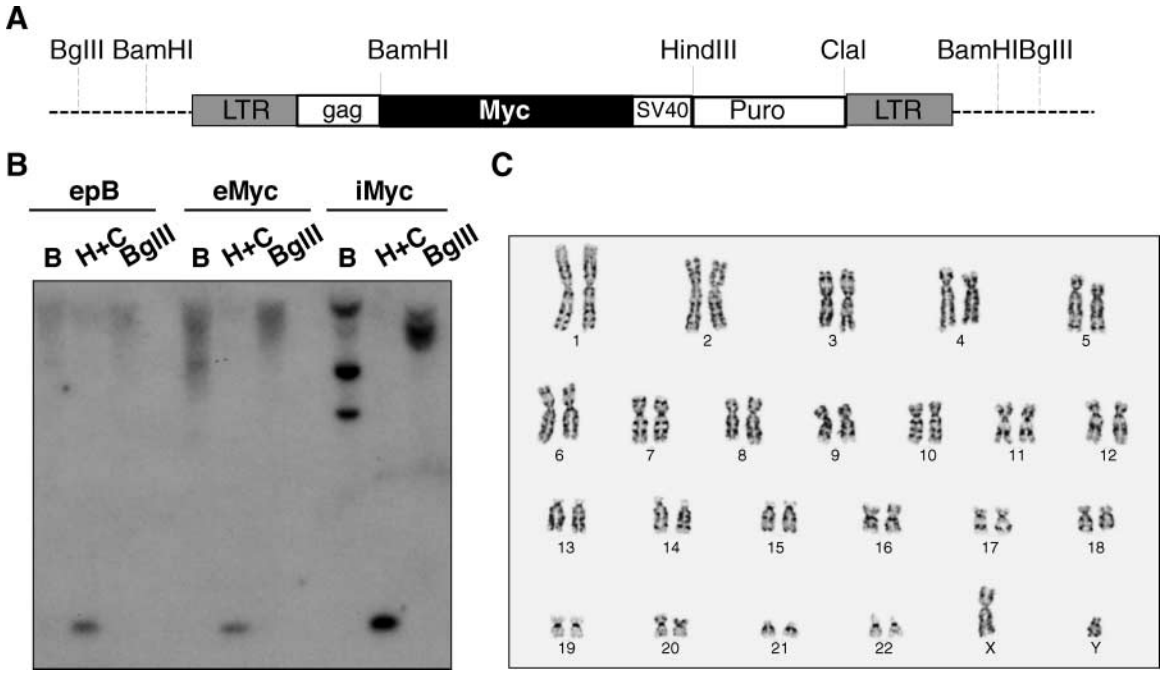

FIGURE 3. c-Myc–immortalized HFFs are oligoclonal. **A.** Schematic diagram of an integrated provirus with the relevant enzymes. **B.** Southern blot analysis of the number of proviral integrations present in the cell population. Early-passage control (*epB*), early-passage c-Myc–expressing (*eMyc*), and c-Myc–immortalized (*iMyc*) cells were analyzed. Lanes show digests with a single-cutter enzyme (*B*), no-cutter enzyme (*BglII*), and enzymes that cut the puromycin fragment (*H+C*). **C.** Representative karyotype of c-Myc–immortalized cells.

c-Myc–Immortalized HFFs Have Reduced Levels of ARF Expression

Although p53 in c-Myc–immortalized HFFs can be activated in response to DNA damage, p53 can also be activated by aberrant expression of oncogenes through the tumor suppressor ARF (14, 20). Because rodent cells immortalized by c-Myc show loss of ARF, we examined *ARF* expression in c-Myc–immortalized HFFs using reverse transcription-PCR (RT-PCR) analysis. *ARF* mRNA was markedly decreased in the cells immortalized by c-Myc, with three of four cell lines showing complete down-regulation of *ARF* (Fig. 6). We also observed increased ARF in early-passage c-Myc–expressing cells relative to control cells, indicating that, similarly to rodent cells, ARF is induced in response to c-Myc (13).

Epigenetic Down-Regulation of ARF in c-Myc–Immortalized HFFs

Both *p16* and *ARF* are gene products of the *INK4a/ARF* locus, but *ARF* is encoded by an alternate reading frame under a different promoter than *p16* (31). Because p16 was actually

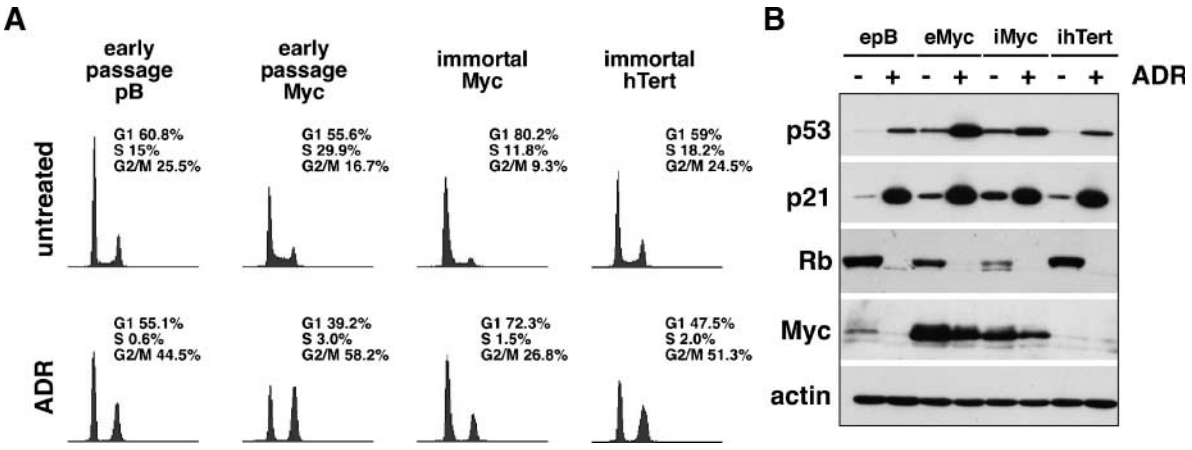

FIGURE 4. c-Myc–immortalized HFFs arrest in response to DNA damage. **A.** Early-passage control (*early passage pB*), early-passage c-Myc–expressing (*early passage Myc*), c-Myc–immortalized (*immortal Myc*), and hTert-immortalized (*immortal hTert*) HFFs were treated with 100 nmol/L Adriamycin (*ADR*) for 24 h and analyzed by flow cytometry. Histograms of propidium iodide–stained cells and proportions of cells in G_1, S, and G_2-M phases of the cell cycle. **B.** Western blot analysis of protein lysates from early-passage control (*epB*), early-passage c-Myc–expressing (*eMyc*), c-Myc–immortalized (*iMyc*), and hTert-immortalized (*ihTert*) HFFs with (+) and without (–) Adriamycin treatment, harvested in parallel with cells analyzed in **A**. Levels of p53, p21, Rb, c-Myc, and actin.

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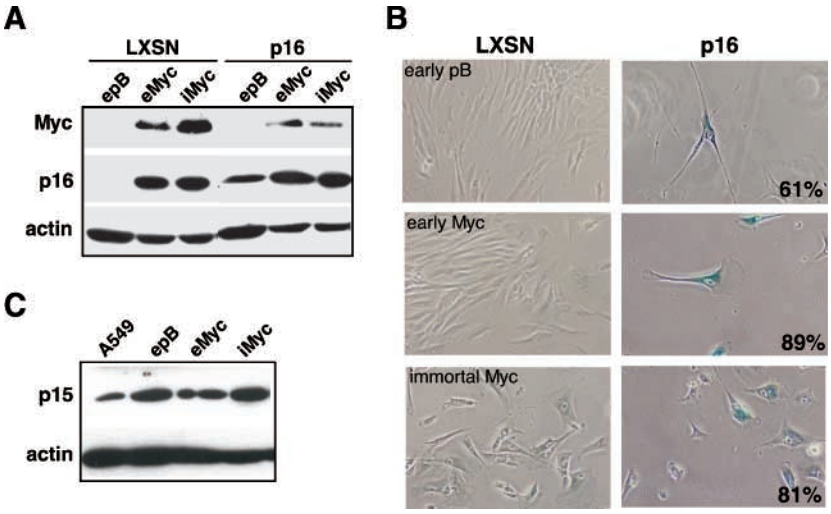

FIGURE 5. c-Myc–immortalized HFFs senesce in response to p16 overexpression and retain expression of p15. **A.** Western blot analysis of protein lysates from early-passage control (*epB*), early-passage c-Myc–expressing (*eMyc*), and c-Myc–immortalized (*iMyc*) cells transduced with empty vector (*LXSN*) or p16 (*p16*). Levels of c-Myc, p16, and actin. **B.** SA β-gal staining of cells described in **A**. Percentages of cells staining positive. **C.** Western blot analysis of protein lysates from A549, early-passage control (*epB*), early-passage c-Myc–expressing (*eMyc*), and c-Myc–immortalized (*iMyc*) cells. Levels of p15 and actin. Lysate from A549 cells served as a positive control for p15.

increased (Fig. 1C) and there were no major chromosomal deletions observed in c-Myc–immortalized HFFs (Fig. 3C), it was unlikely that deletion of the *INK4a/ARF* locus was responsible for the decrease in ARF. We therefore sought to determine if down-regulated *ARF* expression in c-Myc–immortalized HFFs was due to epigenetic modification of the *ARF* promoter. Cells were treated with the demethylating agent 5azaCdR. RT-PCR analysis showed that *ARF* expression was restored 24 h after addition of 5azaCdR (Fig. 7A). The methylation status of the *ARF* gene was confirmed by methylation-specific PCR (Fig. 7B). Significantly, *ARF* was methylated in c-Myc–immortalized but not early-passage c-Myc–expressing cells, indicating that methylation of *ARF* was an event that occurred during selection of the immortalized population. In contrast, *p16* was unmethylated in both early-passage and immortalized cells.

Interestingly, during 5azaCdR treatment, c-Myc–immortalized cells were observed to have a slowed growth rate and larger, flatter morphology compared with untreated controls. In contrast, the growth rate was similar between treated and untreated cells of the other cell populations. A larger fraction of c-Myc–immortalized cells treated with 5azaCdR stained positive for SA β-gal activity (Fig. 7C and D). Although the increase was not dramatic, the percentage of senescent cells did not increase in the other cell lines similarly treated (Fig. 7C).

To determine the consequence of increased ARF expression in c-Myc–immortalized HFFs, we transduced all cell populations with ARF-expressing retroviruses (Fig. 8A). On overexpression of ARF, all cell lines showed decreased rates of cell proliferation, cells with senescent morphology, and cells that stained positive for SA β-gal activity (Fig. 8B). These observations are consistent with our hypothesis that down-regulation of ARF is necessary for continued proliferation of c-Myc–immortalized HFFs.

Discussion

We have shown that constitutive c-Myc overexpression reproducibly immortalizes HFFs isolated from several independent donors. In rodent cells and other strains of human fibroblasts, acute activation of the MycER chimera caused the appearance of karyotypic abnormalities, including gross chromosomal rearrangements (32, 33). c-Myc–immortalized HFFs exhibited a normal karyotype (Fig. 3C), although they did show signs of increased DNA damage as seen by staining with anti-γ-H2AX foci (data not shown), indicating that these cells can efficiently repair genomic damage. Because stable chromosomal rearrangements were not selected for during immortalization (Fig. 3C), we hypothesize that cells with karyotypic abnormalities are likely not favored for survival. c-Myc–immortalized human prostate epithelial cells (24), similar to our findings, had no karyotypic abnormalities; however, they had lost the ability to respond to p16-mediated arrest. This finding is different from our observation (Fig. 5), and the difference might be due to the known requirement of epithelial cells to suppress the p16 pathway to achieve immortalization (34-36). Our result is similar to the observation that in rodent cells Myc expression enables cell growth despite the presence of p16 (37).

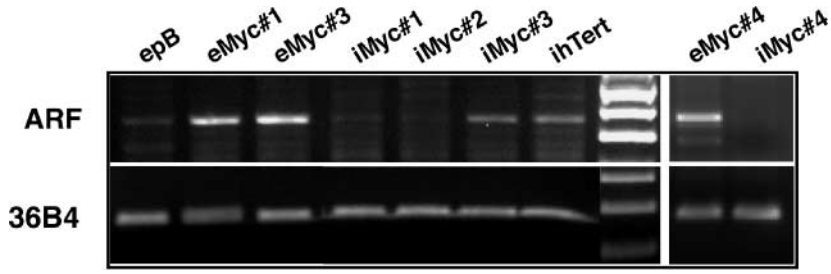

FIGURE 6. c-Myc–immortalized HFFs exhibit decreased ARF expression. RT-PCR analysis for *ARF* expression in early-passage control (*epB*), early-passage c-Myc–expressing (*eMyc*), c-Myc–immortalized (*iMyc*), and hTert-immortalized (*ihTert*) cell lines. 36B4 served as a positive control.

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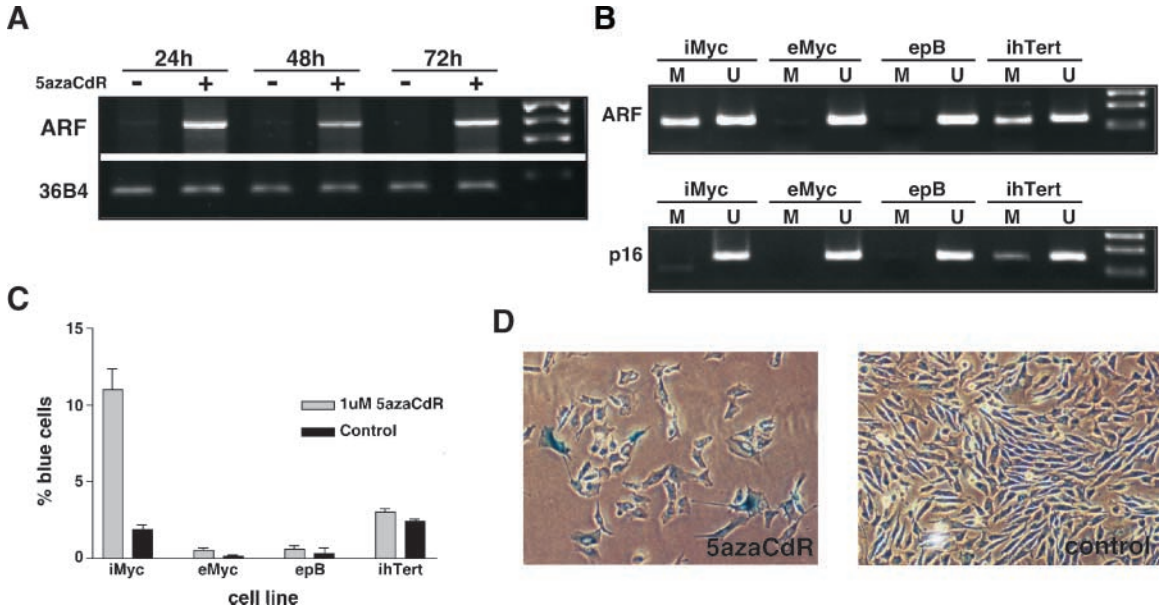

FIGURE 7. Epigenetic down-regulation of *ARF* in c-Myc–immortalized HFFs. **A.** c-Myc–immortalized cells were treated with 1 μmol/L 5azaCdR. RT-PCR analysis for *ARF* expression in treated (+) and untreated (–) cells at the indicated times. 36B4 served as a positive control. **B.** Methylation-specific PCR to confirm methylation status of *ARF* and *p16* in immortalized (*iMyc*) and early-passage (*eMyc*) c-Myc–expressing, early-passage control (*epB*), and hTert-immortalized (*ihTert*) cells. PCR products are specific for the methylated (*M*) or unmethylated (*U*) gene. **C.** Early-passage control (*epB*), early-passage c-Myc–expressing (*eMyc*), c-Myc–immortalized (*iMyc*), and hTert-immortalized (*ihTert*) HFFs were treated with 1 μmol/L 5azaCdR for 72 h and stained for SA β-gal. Quantification of SA β-gal staining. Results for three experiments. **D.** Representative images of c-Myc–immortalized cells stained for SA β-gal.

Importantly, c-Myc–immortalized HFFs underwent a selection step as indicated by the inflection point seen in our growth curve (Fig. 1A) and the oligoclonality of the population (Fig. 3B). One factor that may play a role in the selection process is an optimal level of c-Myc expression, which might be determined by changing pressures in culture. At early passage, cells expressing high levels of c-Myc are likely favored in the culture, as c-Myc–overexpressing cells proliferate more rapidly than controls (data not shown). However, because c-Myc overexpression induces p53 and p16 as well as genomic instability (7, 32), selection then shifts against high levels of c-Myc. When cells reach late passage and telomeres become critically short, increased expression of hTert, and therefore c-Myc, is required to bypass senescence and continue proliferating. The fluctuations observed in levels of c-Myc and telomerase activity support this model, although c-Myc and telomerase levels did not directly correlate with each other (Fig. 1B and C). Telomerase activity may be modulated by a superimposed regulation independent of c-Myc. Furthermore, the oligoclonality of the c-Myc–immortalized cells indicates that other events may have occurred to facilitate immortalization in HFFs.

Interestingly, c-Myc–immortalized HFFs showed a marked reduction of ARF (Fig. 6). In c-Myc–immortalized rodent cells that inactivated the ARF/p53 pathway, loss of functional p53 was a more frequent event, and the few immortalized clones that exhibited ARF loss had only one wild-type allele of *ARF* before introduction of c-Myc that underwent deletion (13). Several human cancers that have lost functional ARF (15) have frequently also lost p16, an event that did not occur in our cells as they retained a functional p16 pathway. This difference might be due to the fact that many of these cancers are epithelial in origin. Additionally, this differential expression of ARF and p16 in c-Myc–immortalized HFFs suggests that loss of ARF may be more necessary than loss of p16 in c-Myc–induced immortalization.

We determined that down-regulated ARF expression in c-Myc–immortalized HFFs was due to methylation. Methylation at the *ARF* promoter to repress gene expression has been observed in some tumor cell lines, particularly colorectal cancers (38-40), but those cell lines also have down-regulated p16 expression. hTert-immortalized HFFs also showed partial methylation of both *ARF* and *p16* (Fig. 7B), possibly indicating the presence of a minority subpopulation of cells carrying methylation at the *INK4a/ARF* locus. However, hTert-immortalized cells treated with 5azaCdR did not show slowed growth or stain positive for SA β-gal activity (compared with untreated; Fig. 7C; data not shown), indicating that methylation of *ARF* does not seem necessary for their continued proliferation.

Despite loss of ARF, c-Myc–immortalized HFFs possessed functional p53 as confirmed by the induction of p21 following DNA damage (Fig. 4B), consistent with the previous observation that ARF is not involved in the p53-dependent DNA damage response (16, 17, 41). However, this contrasts with previous findings that expression of c-Myc prevented induction of p21 following DNA damage through its interactions with the Miz-1 protein (42, 43). This discrepancy may be due to the fact that in these studies c-Myc was acutely activated and the inhibition of p21 induction was most pronounced in the context of already immortalized cells.

Recently, ARF has been shown to have a p53-independent direct interaction with c-Myc (44, 45) that abrogates the ability of c-Myc to activate transcription of its target genes while

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keeping its ability to repress genes intact. Coupled with the observation that c-Myc overexpression up-regulates ARF and activates an apoptotic response in cells (13), these findings establish a potential role for ARF as a specific inhibitor of c-Myc function. Therefore, in c-Myc–overexpressing cells, inactivating ARF would be advantageous to allow c-Myc to activate genes for survival.

Our findings suggest that loss of ARF may be a more favorable cooperating event than loss of p53 or p16 in immortalization of HFFs by c-Myc. However, loss of ARF is not sufficient, as knockdown of ARF by small interfering RNA does not bypass the inflection point observed in c-Myc–overexpressing HFFs (Supplementary Fig. S2). This is consistent with the observation that, in a c-Myc transgenic mouse model, inactivation of ARF seemed necessary but insufficient to induce lymphomagenesis (46, 47).

The fact that c-Myc reproducibly allows establishment of immortalized cells argues that either multiple genetic events are able to collaborate with c-Myc overexpression or more likely that epigenetic events, perhaps of more frequent occurrence or favored by c-Myc, are responsible for the establishment of immortality. In fact, Myc has been shown to interact with the DNA methyltransferase Dnmt3a (48). Because 5azaCdR is a general demethylating agent, it is possible that other genes were down-regulated by methylation in c-Myc–immortalized HFFs, and expression of these genes was also restored after treatment. We do not exclude the contribution of other genetic or epigenetic changes, as multiple events may cooperate to achieve immortalization. However, the finding that restoration of endogenous *ARF* (Fig. 7A) or overexpression of ARF by a retroviral vector (Fig. 8A) resulted in decreased proliferation and increased senescence of c-Myc–immortalized HFFs suggests that down-regulation of ARF is a crucial event in immortalization of c-Myc–expressing HFFs. Finally, these findings indicate a potential role for c-Myc to induce epigenetic changes that contribute to its immortalizing function.

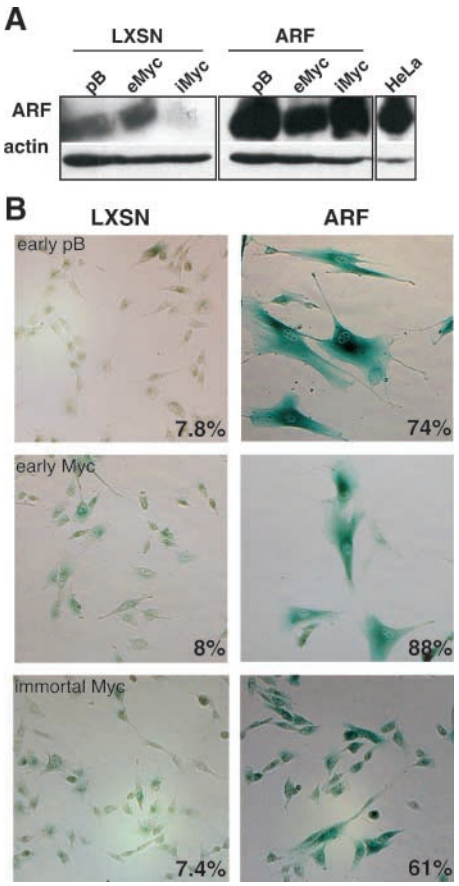

FIGURE 8. Overexpression of ARF in c-Myc–immortalized HFFs induces senescence. **A.** Western blot analysis of protein lysates from early-passage control (*epB*), early-passage c-Myc–expressing (*eMyc*), and c-Myc–immortalized (*iMyc*) cells transduced with empty vector (*LXSN*) or ARF (*ARF*). Levels of ARF and actin. A longer exposure of the blot of LXSN lysate (*left*) is shown to better visualize endogenous levels of ARF, which correlated with mRNA levels observed in Fig. 6. Lysate from HeLa cells served as a positive control for ARF. **B.** SA β-gal staining of cells described in **A**. Percentages of cells staining positive.

Materials and Methods

Cell Culture and Retroviral Infection

HFF strains were isolated from neonatal human foreskins and considered at population doubling level zero when a confluent plate of cells was obtained. Cells were split 1:4 as needed and the population doubling level count increased by two at each split. All cells were cultured in DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin. Retroviruses carrying designated vectors were produced and concentrated as described previously (49). Retroviral vectors (pBABE, pBABE/Myc, LXSN, LXSN/hTert, LXSN/p16, and LXSN/p14ARF) have been described elsewhere (7, 28, 50).

Western Blotting

Preparation of whole-cell lysates and Western blotting were done as described previously (50). Forty micrograms of protein (except for HeLa lysate, 20 μg) were analyzed with antibodies to c-Myc (C-33, Santa Cruz Biotechnology), p53 (antibody 6, Oncogene Research Products), p16 (BD PharMingen), p21 (Waf-1 antibody 1, Oncogene Research Products), Rb (BD PharMingen), p15 (C-20, Santa Cruz Biotechnology), ARF (A300-340A, Bethyl Laboratories), and actin (I-19, Santa Cruz Biotechnology).

Telomerase Activity

Telomerase activity was measured using the TRAPeze telomerase detection kit (Serologicals Corp.) and quantified per kit instructions.

SA β-Gal Staining

Staining was done as described previously (28). Percentages of cells that stained positive were calculated by counting 1,000 cells in random fields per cell line.

Small Interfering RNA Transfection

Small interfering RNA against luciferase and c-Myc (sense strand sequences for siLuc: CGUACGCGGAAUACUUCGATT; siMyc#1: GCUUGUACCUGCAGGAUCUTT; and siMyc#2: CGAUGUUGUUUCUGUGGAATT) was transfected using the Lipofectamine RNAiMAX reagent (Invitrogen).

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Southern Blot Analysis

Genomic DNA (40 μg) was digested with the indicated restriction enzymes overnight. DNA was separated by agarose gel electrophoresis and transferred to a nylon membrane by capillary transfer. The blot was probed with the puromycin gene sequence specific for the proviral DNA corresponding to the retroviral vector pBABE-puro.

Karyotype Analysis

Early-passage pB cells, early-passage c-Myc cells, and immortalized c-Myc cells were treated with colcemid for 40 min and processed for karyotype analysis by the Cytogenetic Lab at the Fred Hutchinson Cancer Research Center. Twenty metaphases for each cell line were analyzed.

Cell Cycle Analysis

For DNA damage response experiments, cells were treated with 100 nmol/L Adriamycin (Calbiochem) as indicated. Cells were then trypsinized, fixed, and analyzed as described previously (50).

ARF *Expression Analysis*

Cellular RNA was isolated with Trizol (Invitrogen) and purified using RNeasy Mini kit (Qiagen). Reverse transcription reactions were done using SuperScript II (Life Technologies) and random hexamers (Invitrogen). Hot-start RT-PCR was done using primers for *ARF* (51) with conditions as follows: 95°C for 5 min, 5 cycles of (94°C for 30 s, 58°C for 30 s, 72°C for 30 s), 30 cycles of (94°C for 30 s, 54°C for 30 s, 72°C for 30 s), and 72°C for 2 min. PCR product was visualized on a 1.5% agarose gel. For restoration of ARF expression, cells were sparsely plated at equal density and allowed to grow overnight before being treated with 1 μmol/L of freshly prepared 5azaCdR (Sigma). For each treated plate, one plate of cells was untreated in parallel. RNA was isolated every 24 h and RT-PCR was done as described previously.

Methylation Status of ARF *and* p16

Cells were lysed with Tris-EDTA and 1% SDS, and DNA was extracted by phenol chloroform precipitation. Bisulfite modification was done using the EpiTect Bisulfite kit (Qiagen). Methylation-specific PCR was done using primers for *ARF* (40) and for *p16* (52) and HotStar Taq Plus DNA Polymerase (Qiagen). PCR conditions were as follows: for *ARF*, 95°C for 5 min, 42 cycles of (94°C for 30 s, 54°C for 30 s, 72°C for 30 s), and 72°C for 4 min; for *p16*, 95°C for 5 min, 40 cycles of (94°C for 30 s, 56°C for 30 s, 72°C for 30 s), and 72°C for 4 min. PCR product was visualized on a 2% agarose gel.

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